

## BRANCHED MOLECULAR SCAFFOLDS FOR LINKING POLYMER RESIDUES TO BIOLOGICALLY ACTIVE MOIETIES

5 The present invention relates to branched molecular scaffolds which are capable of linking two polymer residues (derived, for example, from polyethylene glycol) to two, three or four residues derived from biologically active molecules. Methods for the production of such molecules, and pharmaceutical compositions containing them, are also provided.

10 The hydrophilic polymer polyethylene glycol (PEG) has been covalently attached to biologically active molecules for a variety of reasons, including to increase water solubility (Greenwald *et al.*, *J. Org. Chem.*, 1995, **60**, 331-336), to extend the circulating half-life and to reduce immunogenicity (Chapman, *Advanced Drug Delivery Reviews*, 2002, **54**, 531-545). In general, site-specific attachment of PEG molecules is preferable to random attachment, which can adversely affect the biological activity of the molecule.

15 In order to attach a high enough molecular weight of PEG to the biologically active molecule, branched PEG molecules have been used in place of very high molecular weight linear polymer chains which can be difficult to prepare, as well as being expensive.

20 For various reasons, it is considered advantageous to attach more than one biologically active molecule to a single branched PEG scaffold. In such instances, for example, a substantial increase in potency can be observed.

Branched PEG polymers comprising a single site for the attachment of one or more biologically active molecules are described, for example, in US patents 6,362,254 and 5,932,462.

25 US 6,251,382 provides a branched scaffold comprising *inter alia* a multiplicity of separate sites for attachment of at least two polymer chains and at least two biologically active molecules. The molecules described therein are stated to be biodegradable, polymer-based conjugates having the following formula:

30 
$$(D)_n-M-(R_1)_m$$

wherein

(m) and (n) independently represent positive integers, preferably from about 1 to about 6 each;

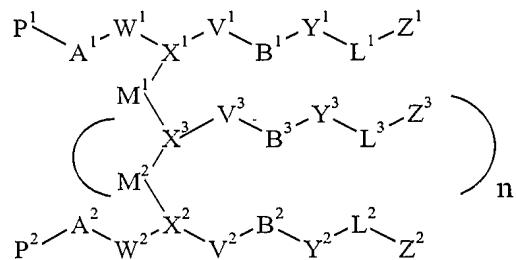
D is a residue of a biologically active moiety;

M is a multifunctional linker/spacer moiety; and

5 R<sub>1</sub> is a polymer residue.

The present invention provides new branched molecular scaffolds which are capable of linking two polymer residues (derived, for example, from PEG) to two, three or four residues derived from biologically active molecules, the latter being attached to the scaffold by means of hydrolytically stable linkages.

10 Thus, the present invention provides a compound of formula (I):



(I)

wherein

15 P<sup>1</sup> and P<sup>2</sup> independently represent a polymer residue;

Z<sup>1</sup>, Z<sup>2</sup> and Z<sup>3</sup> independently represent the residue of a biologically active moiety;

X<sup>1</sup>, X<sup>2</sup> and X<sup>3</sup> independently represent CR<sup>1</sup> or N;

A<sup>1</sup> and A<sup>2</sup> independently represent -CONH-, -NHCO-, -OC(O)N(R<sup>2</sup>)-, -N(R<sup>2</sup>)C(O)O- or -NHCONH-;

20 B<sup>1</sup>, B<sup>2</sup> and B<sup>3</sup> independently represent -CONH- or -CO-;

V<sup>1</sup> and V<sup>2</sup> independently represent a covalent bond or -(CH<sub>2</sub>)<sub>v</sub>-;

W<sup>1</sup> and W<sup>2</sup> independently represent a covalent bond or -(CH<sub>2</sub>)<sub>w</sub>-;

Y<sup>1</sup>, Y<sup>2</sup> and Y<sup>3</sup> independently represent -(CH<sub>2</sub>)<sub>y</sub>-;

L<sup>1</sup>, L<sup>2</sup> and L<sup>3</sup> independently represent a spacer group;

25 M<sup>1</sup> and M<sup>2</sup> independently represent a covalent bond or -(CH<sub>2</sub>)<sub>m</sub>-;

R<sup>1</sup> represents hydrogen or C<sub>1-4</sub> alkyl;

R<sup>2</sup> represents hydrogen or C<sub>1-4</sub> alkyl;

n is zero, 1 or 2;  
v is 1, 2, 3 or 4;  
w is 1, 2, 3 or 4;  
y is 1, 2, 3, 4, 5 or 6; and  
5 m is 1, 2 or 3.

The present invention falls within the broadest generic scope of US 6,251,382.

However, there is no specific disclosure therein of a compound falling within the scope of formula (I) as depicted above.

As used herein, the term “C<sub>1-4</sub> alkyl” refers to straight-chained and branched alkyl 10 groups containing 1 to 4 carbon atoms. Such groups are methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl and *tert*-butyl.

As used herein, the term “residue” will be understood to mean that portion of a polymer or of a biologically active moiety which remains after it has undergone a substitution reaction as such terminology is familiar to the person skilled in the art.

15 The polymer residues P<sup>1</sup> and P<sup>2</sup> in the compounds of formula (I) above will suitably be residues of substantially water-soluble, substantially non-antigenic polymers as described, for example, in US 6,251,382 B1, with particular reference to the passage running from column 16, line 52 to column 18, line 14, the contents of which are herein incorporated by reference. Typical polymers of which P<sup>1</sup> and P<sup>2</sup> are residues include 20 polyalkylene oxides such as polyethylene glycols (PEGs). As regards attaching PEG moieties in general, reference is made to “Poly(ethyleneglycol) Chemistry, Biotechnical and Biomedical Applications”, 1992, J. Milton Harris (ed), Plenum Press, New York; “Poly(ethyleneglycol) Chemistry and Biological Applications”, 1997, J. Milton Harris and S. Zalipsky (eds), American Chemical Society, Washington DC; and “Bioconjugation 25 Protein Coupling Techniques for the Biomedical Sciences”, 1998, M. Aslam and A. Dent, Grove Publishers, New York. Particular PEG molecules include 20K methoxy-PEG-amine (obtainable from Nektar, formerly Shearwater; Rapp Polymere; and SunBio) and M-PEG-SPA (obtainable from Nektar, formerly Shearwater).

Suitably, P<sup>1</sup> and P<sup>2</sup> are identical.

30 In the compounds of formula (I) above, the residues Z<sup>1</sup>, Z<sup>2</sup> and Z<sup>3</sup> will suitably be residues of the entities referred to in, for example, US 6,251,382 B1, with particular reference to the passage running from column 18, line 15 to column 22, line 67, the contents of which are herein incorporated by reference. Typical biologically active

moieties of which  $Z^1$ ,  $Z^2$  and  $Z^3$  are residues include antibodies and antibody fragments such as those referred to in US 6,251,382 B1, column 22, lines 14-22.

Thus, the residues  $Z^1$ ,  $Z^2$  and  $Z^3$  include whole antibodies and functionally active fragments or derivatives thereof and may be, but are not limited to, polyclonal, 5 monoclonal, multi-valent, multi-specific, humanized or chimeric antibodies, single chain antibodies, Fab fragments, Fab' and  $F(ab')_2$  fragments and epitope-binding fragments of any of the above.

Antibodies include immunoglobulin molecules and immunologically active 10 portions of immunoglobulin molecules, i.e. molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any class (e.g. IgG, IgE, IgM, IgD or IgA) or subclass of immunoglobulin molecule.

Monoclonal antibodies may be prepared by any method known in the art such as the hybridoma technique (Kohler & Milstein, *Nature*, 1975, **256**, 495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today*, 15 1983, **4**, 72) and the EBV-hybridoma technique (Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy", pp. 77-96, Alan R. Liss, Inc., 1985).

Antibodies for use in the invention may also be generated using single lymphocyte 20 antibody methods by cloning and expressing immunoglobulin variable region cDNAs generated from single lymphocytes selected for the production of specific antibodies by, for example, the methods described by Babcock, J. *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, **93**(15), 7843-7848, and in WO 92/02551.

Humanized antibodies are antibody molecules from non-human species having 25 one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule (see, for example, US 5,585,089).

Chimeric antibodies are those antibodies encoded by immunoglobulin genes that have been genetically engineered so that the light and heavy chain genes are composed of immunoglobulin gene segments belonging to different species. These chimeric antibodies are likely to be less antigenic. Bivalent antibodies may be made by methods known in the 30 art (Milstein *et al.*, *Nature*, 1983, **305**, 537-539; WO 93/08829; Traunecker *et al.*, *EMBO J.*, 1991, **10**, 3655-3659). Multi-valent antibodies may comprise multiple specificities or may be monospecific (see, for example, WO 92/22853).

The antibodies for use in the present invention can also be generated using various phage display methods known in the art and include those disclosed by Brinkman *et al.*, *J. Immunol. Methods*, 1995, **182**, 41-50; Ames *et al.*, *J. Immunol. Methods*, 1995, **184**, 177-186; Kettleborough *et al.* *Eur. J. Immunol.*, 1994, **24**, 952-958; Persic *et al.*, *Gene*, 1997 **187**, 9-18; and Burton *et al.*, *Advances in Immunology*, 1994, **57**, 191-280; WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; and WO 95/20401; and US 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743; and 5,969,108. Techniques for the production of single chain antibodies, such as those described in US 4,946,778, can also be adapted to produce single chain antibodies. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

Particular antibody fragments include those described in international patent applications PCT/GB2004/002810, PCT/GB2004/002870 and PCT/GB2004/002871 (all filed on 1 July 2004), claiming priority variously from United Kingdom patent applications 0315450.7, 0315457.2 (both filed on 1 July 2003) and 0319588.0 (filed on 20 August 2003).

Suitably,  $Z^1$  and  $Z^2$  are identical.

In one embodiment,  $X^1$  represents  $CR^1$ . In another embodiment,  $X^1$  represents  $N$ .

20 In one embodiment,  $X^2$  represents  $CR^1$ . In another embodiment,  $X^2$  represents  $N$ .

In one embodiment,  $X^3$  represents  $CR^1$ . In another embodiment,  $X^3$  represents  $N$ .

Suitably,  $X^1$  and  $X^2$  are identical.

Suitably,  $A^1$  represents -CONH- or -NHCO-. In one embodiment,  $A^1$  represents -CONH-. In another embodiment,  $A^1$  represents -NHCO-.

25 Suitably,  $A^2$  represents -CONH- or -NHCO-. In one embodiment,  $A^2$  represents -CONH-. In another embodiment,  $A^2$  represents -NHCO-.

Suitably,  $A^1$  and  $A^2$  are identical.

In one embodiment,  $B^1$  represents -CONH-. In another embodiment,  $B^1$  represents -CO-. Where  $B^1$  represents -CONH-,  $X^1$  typically represents CH. Where  $B^1$  represents -CO-,  $X^1$  typically represents N.

In one embodiment,  $B^2$  represents -CONH-. In another embodiment,  $B^2$  represents -CO-. Where  $B^2$  represents -CONH-,  $X^2$  typically represents CH. Where  $B^2$  represents -CO-,  $X^2$  typically represents N.

In one embodiment,  $B^3$  represents -CONH-. In another embodiment,  $B^3$  represents -CO-. Where  $B^3$  represents -CONH-,  $X^3$  typically represents CH. Where  $B^3$  represents -CO-,  $X^3$  typically represents N.

Suitably,  $B^1$  and  $B^2$  are identical.

5 In a preferred embodiment,  $V^1$  represents a covalent bond. In another embodiment,  $V^1$  represents  $-(CH_2)_v-$  in which v is as defined above.

In a preferred embodiment,  $V^2$  represents a covalent bond. In another embodiment,  $V^2$  represents  $-(CH_2)_v-$  in which v is as defined above.

10 In a preferred embodiment,  $V^3$  represents a covalent bond. In another embodiment,  $V^3$  represents  $-(CH_2)_v-$  in which v is as defined above.

Suitably,  $V^1$  and  $V^2$  are identical.

In one embodiment,  $W^1$  represents a covalent bond. In another embodiment,  $W^1$  represents  $-(CH_2)_w-$  in which w is as defined above.

15 In one embodiment,  $W^2$  represents a covalent bond. In another embodiment,  $W^2$  represents  $-(CH_2)_w-$  in which w is as defined above.

Suitably,  $W^1$  and  $W^2$  are identical.

Suitably,  $Y^1$  and  $Y^2$  are identical.

20 The spacer groups  $L^1$ ,  $L^2$  and  $L^3$  will suitably comprise any moiety familiar to the person skilled in the art which is capable of forming a bridge between the alkylene chain  $Y^1$ ,  $Y^2$  and (where present)  $Y^3$  and the residue  $Z^1$ ,  $Z^2$  and (where present)  $Z^3$  respectively. For example, where  $Z^1$  and/or  $Z^2$  and/or  $Z^3$  is the residue of a polypeptide molecule (e.g. an antibody or a fragment thereof) containing a cysteine residue the corresponding spacer group  $L^1$  and/or  $L^2$  and/or  $L^3$  will suitably be a maleimide residue, which may be covalently linked to the cysteine-containing polypeptide residue  $Z^1$  and/or  $Z^2$  and/or  $Z^3$  via a thiol linkage and to the alkylene chain  $Y^1$  and/or  $Y^2$  and/or  $Y^3$  through the maleimide nitrogen atom.

Suitably,  $L^1$  and  $L^2$  are identical.

In one embodiment,  $M^1$  represents a covalent bond. In another embodiment,  $M^1$  represents  $-(CH_2)_m-$  in which m is as defined above.

30 In one embodiment,  $M^2$  represents a covalent bond. In another embodiment,  $M^2$  represents  $-(CH_2)_m-$  in which m is as defined above.

In a preferred embodiment,  $R^1$  is hydrogen. In another embodiment,  $R^1$  represents  $C_{1-4}$  alkyl, especially methyl.

In a preferred embodiment,  $R^2$  is hydrogen. In another embodiment,  $R^2$  represents  $C_{1-4}$  alkyl, especially methyl.

Suitably,  $n$  is zero or 1.

In a preferred embodiment,  $n$  is zero, in which case  $M^1$  is directly attached to  $X^2$ .

5 In another embodiment,  $n$  is 1. In a further embodiment,  $n$  is 2.

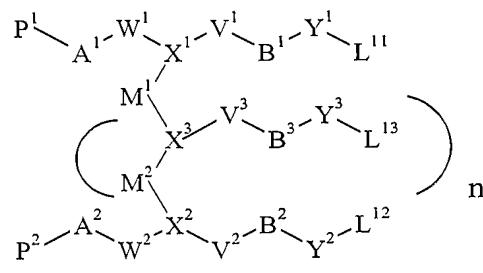
In one embodiment,  $w$  is 1. In another embodiment,  $w$  is 2. In an additional embodiment,  $w$  is 3. In a further embodiment,  $w$  is 4. Favourably,  $w$  is 1 or 2.

In one embodiment,  $y$  is 1. In another embodiment,  $y$  is 2. In an additional embodiment,  $y$  is 3. In a further embodiment,  $y$  is 4. In a still further embodiment,  $y$  is 5.

10 In a yet further embodiment,  $y$  is 6. Favourably,  $y$  is 2, 3 or 4, typically 2 or 4.

In one embodiment,  $m$  is 1. In another embodiment,  $m$  is 2. In an additional embodiment,  $m$  is 3. Favourably,  $m$  is 2.

In another aspect, the present invention provides novel scaffold molecules which are valuable intermediates for the attachment of the biologically active moieties of which 15  $Z^1$ ,  $Z^2$  and  $Z^3$  are residues. Thus, the invention also provides a compound of formula (II):



(II)

wherein

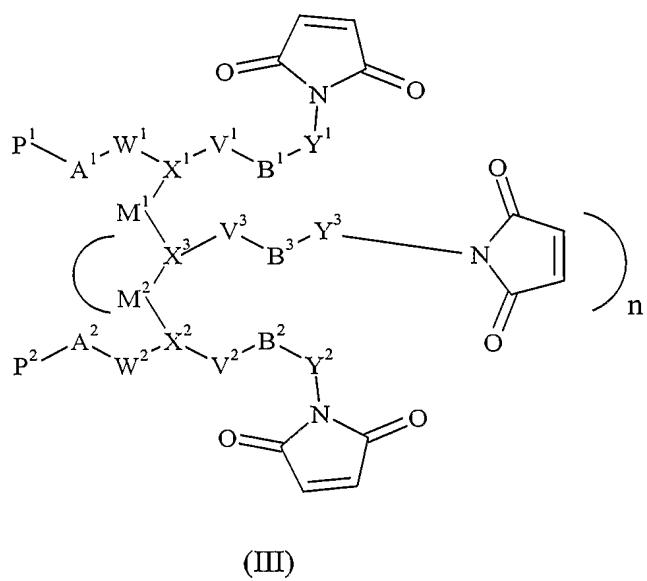
20  $L^{11}$ ,  $L^{12}$  and  $L^{13}$  represent groups capable of attaching the residue  $Z^1$ ,  $Z^2$  and  $Z^3$  respectively, or capable of being converted into such groups; and

each of the other variables is as defined above in relation to formula (I).

Where  $Z^1$  and/or  $Z^2$  and/or  $Z^3$  is the residue of a polypeptide molecule (e.g. an antibody or a fragment thereof), the corresponding group  $L^1$  and/or  $L^2$  and/or  $L^3$  may be 25 attached to the polypeptide through any available amino acid side-chain or terminal amino acid functional group located in the antibody fragment, for example any free amino, imino, thiol, hydroxy or carboxyl group. Such amino acids may occur naturally

in, for example, the antibody fragment or may be engineered into the fragment using recombinant DNA methods (see, for example, US 5,219,996). In a preferred aspect of the invention the two groups are covalently linked through a thiol group of a cysteine residue located in the fragment. The covalent linkage will generally be a disulphide bond or a sulphur-carbon bond, preferably the latter. In one example where a thiol group is used as the point of attachment appropriately activated groups, for example thiol-selective derivatives such as maleimide and cysteine derivatives, may be used.

5 In a preferred feature, the groups  $L^{11}$ ,  $L^{12}$  and (where present)  $L^{13}$  are identical and represent maleimide derivatives attached to the remainder of the molecule through the maleimide nitrogen atom. Accordingly, one illustrative subset of the compounds of formula (II) above is represented by the compounds of formula (III):



10 15 wherein each of the variables is as defined above in relation to formula (I).

According to a further aspect of the invention there is provided a pharmaceutical composition which comprises a compound of formula (I) as defined above in association with one or more pharmaceutically acceptable carriers, excipients or diluents.

20 Pharmaceutical compositions according to the invention may take a form suitable for oral, buccal, parenteral, nasal, topical, ophthalmic or rectal administration, or a form suitable for administration by inhalation or insufflation.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets, lozenges or capsules prepared by conventional means with

pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methyl cellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogenphosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents, emulsifying agents, non-aqueous vehicles or preservatives. The preparations may also contain buffer salts, flavouring agents, colouring agents or sweetening agents, as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

15 For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds of formula (I) may be formulated for parenteral administration by injection, e.g. by bolus injection or infusion. Formulations for injection may be presented in unit dosage form, e.g. in glass ampoules or multi-dose containers, e.g. glass vials. The 20 compositions for injection may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising, preserving and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

25 In addition to the formulations described above, the compounds of formula (I) may also be formulated as a depot preparation. Such long-acting formulations may be administered by implantation or by intramuscular injection.

For nasal administration or administration by inhalation, the compounds according to the present invention may be conveniently delivered in the form of an aerosol spray presentation for pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane, fluorotrichloromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas or mixture of gases.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack or dispensing device may be accompanied by instructions for administration.

For topical administration the compounds according to the present invention may 5 be conveniently formulated in a suitable ointment containing the active component suspended or dissolved in one or more pharmaceutically acceptable carriers. Particular carriers include, for example, mineral oil, liquid petroleum, propylene glycol, polyoxyethylene, polyoxypropylene, emulsifying wax and water. Alternatively, the 10 compounds according to the present invention may be formulated in a suitable lotion containing the active component suspended or dissolved in one or more pharmaceutically acceptable carriers. Particular carriers include, for example, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, benzyl alcohol, 2-octyldodecanol and water.

For ophthalmic administration the compounds according to the present invention 15 may be conveniently formulated as microionized suspensions in isotonic, pH-adjusted sterile saline, either with or without a preservative such as a bactericidal or fungicidal agent, for example phenylmercuric nitrate, benzylalkonium chloride or chlorhexidine acetate. Alternatively, for ophthalmic administration compounds may be formulated in an ointment such as petrolatum.

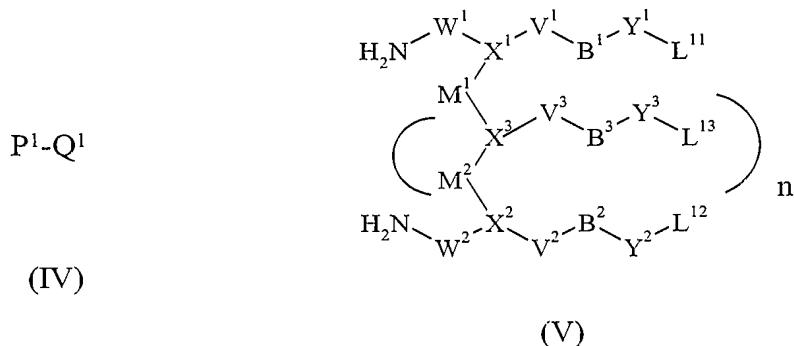
20 For rectal administration the compounds according to the present invention may be conveniently formulated as suppositories. These can be prepared by mixing the active component with a suitable non-irritating excipient which is solid at room temperature but liquid at rectal temperature and so will melt in the rectum to release the active component. Such materials include, for example, cocoa butter, beeswax and polyethylene glycols.

25 The quantity of a compound of the invention required for the prophylaxis or treatment of a particular condition will vary depending on the compound chosen and the condition of the patient to be treated. In general, however, daily dosages may range from around 10 ng/kg to 1000 mg/kg, typically from 100 ng/kg to 100 mg/kg, e.g. around 0.01 mg/kg to 40 mg/kg body weight for oral or buccal administration, from around 10 ng/kg 30 to 50 mg/kg body weight for parenteral administration, and from around 0.05 mg to around 1000 mg, e.g. from around 0.5 mg to around 1000 mg, for nasal administration or administration by inhalation or insufflation.

The compounds of formula (I) may be prepared by a process which comprises attachment of the residues  $Z^1$ ,  $Z^2$  and (where present)  $Z^3$  to the appropriate compound of formula (II). This process may be accomplished using procedures which are well known to the person skilled in the art such as, for example, the methods described in US 5,625,1382 B1, with particular reference to column 23, lines 1 to 50, the contents of which are herein incorporated by reference.

The compounds of formula (II) wherein  $P^1$  and  $P^2$  are identical and  $A^1$  and  $A^2$  are both -CONH- may be prepared by a process which comprises reacting a compound of formula (IV) with a compound of formula (V):

10



wherein  $Q^1$  represents an activated carboxylate moiety; and the remaining variables are as defined above.

15

Examples of activated carboxylate moieties for the substituent  $Q^1$  include acid chlorides; acid anhydrides; and the ester formed when a carboxylic acid ( $Q^1 = -CO_2H$ ) is reacted with *N*-hydroxysuccinimide.

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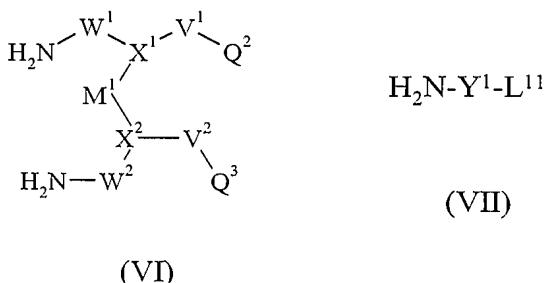
The reaction between compounds (IV) and (V) is conveniently effected in a suitable solvent, e.g. dichloromethane, typically in the presence of an organic base, e.g. triethylamine.

25

The compounds of formula (II) wherein  $P^1$  and  $P^2$  are identical and  $A^1$  and  $A^2$  are both -OC(O)N(H)- may be prepared by a process which comprises reacting a compound of formula  $P^1$ -OC(O)R<sup>x</sup>, wherein R<sup>x</sup> represents a readily displaceable group such as a halogen atom (e.g. chloro), 4-nitrophenoxy or 1-succinimidylxy; with a compound of formula (V) as defined above.

The requisite intermediate of formula  $P^1\text{-OC(O)R}^x$  may be prepared by treating a compound of formula  $P^1\text{-OH}$  with, for example, phosgene, 4-nitrophenyl chloroformate or *N,N'*-disuccinimidyl carbonate.

By way of illustration, the compounds of formula (V) wherein n is zero, B<sup>1</sup> and B<sup>2</sup> are both -CONH-, Y<sup>1</sup> and Y<sup>2</sup> are identical and L<sup>11</sup> and L<sup>12</sup> are identical may be prepared by a process which comprises reacting a compound of formula (VI) with a compound of formula (VII):

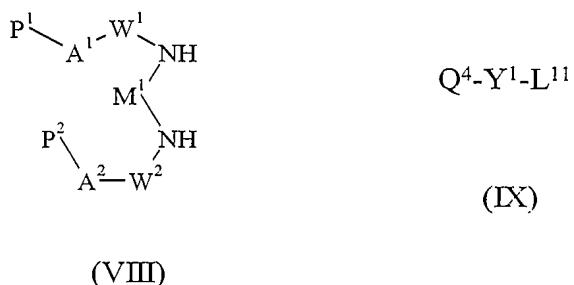


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wherein  $Q^2$  and  $Q^3$  independently represent an activated carboxylate moiety as defined above for  $Q^1$ ; and the remaining variables are as defined above.

The reaction between compounds (VI) and (VII) is conveniently effected in a suitable solvent, e.g. dichloromethane, typically in the presence of an organic base, e.g. triethylamine.

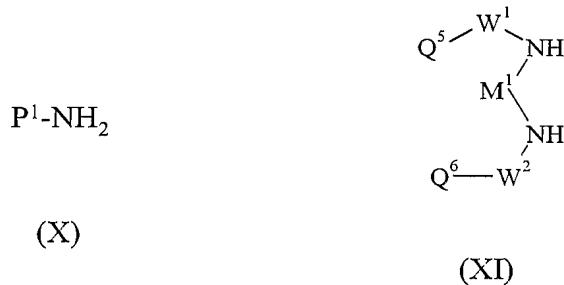
In an alternative procedure, and by way of illustration, the compounds of formula (II) wherein n is zero,  $X^1$  and  $X^2$  are both N,  $B^1$  and  $B^2$  are both -CO-,  $V^1$  and  $V^2$  are both covalent bonds,  $Y^1$  and  $Y^2$  are identical and  $L^{11}$  and  $L^{12}$  are identical may be prepared by a process which comprises reacting a compound of formula (VIII) with a compound of formula (IX):



wherein  $Q^4$  represents an activated carboxylate moiety as defined above for  $Q^1$ ; and the remaining variables are as defined above.

The reaction between compounds (VIII) and (IX) is conveniently effected in a suitable solvent, e.g. dichloromethane, typically in the presence of an organic base, e.g. 5 triethylamine.

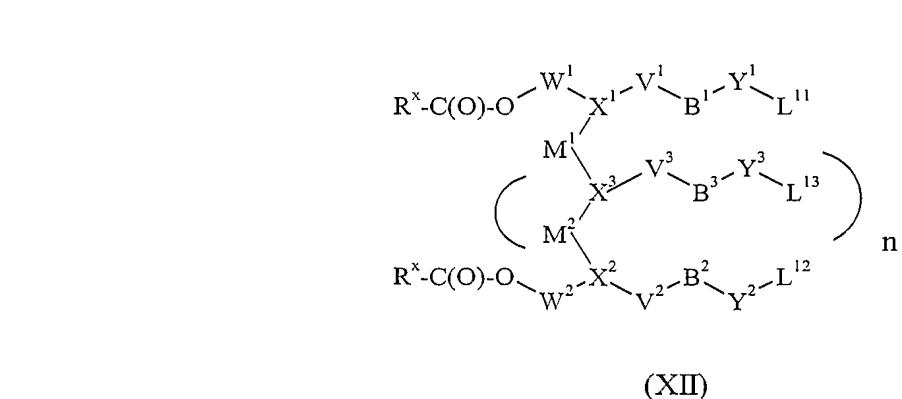
The compounds of formula (VIII) wherein  $P^1$  and  $P^2$  are identical and  $A^1$  and  $A^2$  are both -NHCO- may be prepared by reacting a compound of formula (X) with a compound of formula (XI):



wherein  $Q^5$  and  $Q^6$  independently represent an activated carboxylate moiety as defined above for  $Q^1$ ; and the remaining variables are as defined above.

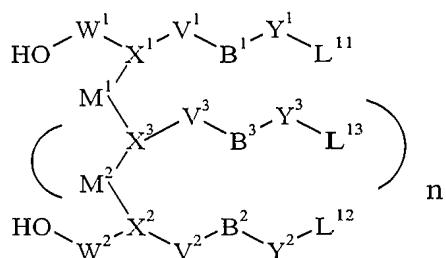
The reaction between compounds (X) and (XI) is conveniently effected in a 15 suitable solvent, e.g. dichloromethane, typically in the presence of an organic base, e.g. triethylamine.

The compounds of formula (II) wherein  $P^1$  and  $P^2$  are identical and  $A^1$  and  $A^2$  are both -N(H)C(O)O- may be prepared by a process which comprises reacting a compound of formula (X) as defined above with a compound of formula (XII):



wherein  $R^x$  and the remaining variables are all as defined above.

The intermediates of formula (XII) may be prepared by treating a compound of formula (XIII):



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(XIII)

wherein the variables are all as defined above; with, for example, phosgene, 4-nitrophenyl chloroformate or *N,N'*-disuccinimidyl carbonate.

Where  $R^2$  is  $C_{1-4}$  alkyl, attachment of the  $R^2$  moiety may be effected by 10 conventional *N*-alkylation procedures.

The compounds of formula (II) wherein  $P^1$  and  $P^2$  are identical and  $A^1$  and  $A^2$  are both  $-NHCONH-$  may be prepared by a process which comprises reacting a compound of formula  $P^1-N=C=O$  with a compound of formula (V) as defined above.

The requisite intermediate of formula  $P^1-N=C=O$  may, for example, be prepared 15 by treating a compound of formula (X) as defined above with phosgene.

Where they are not commercially available, the compounds of formula (IV), (VI), (VII), (IX), (X), (XI) and (XIII) may be prepared by methods analogous to those described in the accompanying Examples, or by standard methods well known from the art.

Where a mixture of products is obtained from any of the processes described 20 above for the preparation of compounds according to the invention, the desired product can be separated therefrom at an appropriate stage by conventional methods such as gel permeation chromatography; cation or anion exchange; preparative HPLC; or column chromatography utilising, for example, silica and/or alumina in conjunction with an appropriate solvent system.

During any of the above synthetic sequences it may be necessary and/or desirable 25 to protect sensitive or reactive groups on any of the molecules concerned. This may be

achieved by means of conventional protecting groups, such as those described in *Protective Groups in Organic Chemistry*, ed. J.F.W. McOmie, Plenum Press, 1973; and T.W. Greene & P.G.M. Wuts, *Protective Groups in Organic Synthesis*, John Wiley & Sons, 3<sup>rd</sup> edition, 1999. The protecting groups may be removed at any convenient 5 subsequent stage utilising methods known from the art.

The following non-limiting Examples illustrate the invention.

### Intermediate 1

#### **(4-Maleimidyl-butyl)-carbamic acid *tert*-butyl ester**

10 To a solution of N-BOC-1,4-diaminobutane (8.324g, 0.044mol) in dry toluene (50ml) was added maleic anhydride (4.336g, 0.044mol) and the solution heated at reflux under Dean-Stark conditions for two days. The solvent was removed and the residue purified by silica column chromatography eluting with 25-40% ethyl acetate in hexane to give the title compound, 1.461g, 12%, as a white solid.

15 m/z (LCMS ES+, 70V) 291 (MH<sup>+</sup>).  
 $\delta_H$  (CDCl<sub>3</sub>) 6.63 (2H, s), 4.46 (1H, br), 3.48 (2H, t, J7.1Hz), 3.08 (2H, m), 1.56 (2H, p, J7.4Hz), 1.41 (2H, m), 1.38 (9H, s).

### Intermediate 2

#### ***meso*-2,3-Bis-*tert*-butoxycarbonylamino-succinic acid**

20 To a solution of *meso*-2,3-diamino succinic acid (1.96g, 0.013mol) and triethylamine (5.36g, 0.053mol) in water (50ml) was added a solution of di-*tert*-butyl dicarbonate (6.35g, 0.029mol) in dioxane (30ml) over a period of 20min. After 2 hours the solvent was reduced to 10ml, diluted with water to 50ml and washed with 25 dichloromethane (4x30ml). The solution was then acidified to pH 1-2 with 2M HCl and extracted into ethyl acetate (5x50ml). The ethyl acetate solution was dried over magnesium sulphate and the solvent removed to yield the title compound, 4.345g, 94%, as a colourless glass.  
m/z (LCMS ES+, 70V) 371 (MNa<sup>+</sup>).  
30  $\delta_H$  (d<sub>6</sub>-DMSO, 370K) 6.03 (2H, br), 4.49 (2H, s), 1.42 (18H, s).

**Intermediate 3****{tert-Butoxycarbonyl-[2-(tert-butoxycarbonyl-carboxymethyl-amino)-ethyl]-amino}-acetic acid**

To a stirred suspension of ethylenediamine-N,N'-diacetic acid (1.00g, 5.68mmol) in methanol (150ml) was added triethylamine (2.30g, 22.7mmol) followed by di-*tert*-butyl dicarbonate (2.48g, 11.4mmol). The suspension was heated at reflux for 10 minutes until the majority of solids had dissolved then allowed to cool to ambient temperature and stirred for 2h. The suspension was filtered, the solvent removed, the residue dissolved in water (40ml) and the pH raised to approximately 8-9 with triethylamine. The solution was washed with dichloromethane (5x40ml), acidified with 1M HCl to a pH of approximately 1 and extracted with DCM (10x40ml) followed by ethyl acetate (10x40ml). The ethyl acetate and dichloromethane fractions were dried over magnesium sulphate, combined and the solvent removed to yield the desired material, 1.558g, 74%, as a white solid.

m/z (LCMS ES+, 70V) 399 (MNa<sup>+</sup>).

$\delta_H$  (d6-DMSO, 380K) 3.86 (4H, s), 3.37 (4H, s), 1.42 (18H, s).

**Intermediate 4****3-(tert-Butoxycarbonyl-[2-[tert-butoxycarbonyl-(2-carboxy-ethyl)-amino]-ethyl]-amino)-propionic acid**

To ethylene diamine-N,N'-dipropionic acid di-hydrochloride salt (2.00g, 7.2mmol) in water (50ml) and triethylamine (4.38g, 43mmol) was added di-*tert* butyl di-carbonate (3.307g, 15mmol) in dioxane (30ml) over a period of 10 minutes. The reaction was left overnight at ambient temperature, the dioxane removed under reduced pressure and the remaining aqueous solution washed with dichloromethane (4x50ml). The aqueous layer was acidified with concentrated HCl to a pH of approximately 1, and the resulting white precipitate extracted into ethyl acetate (5x60ml). The ethyl acetate solution was dried over magnesium sulphate and the solvent removed to yield the product, 2.68g, 92%, as a white solid.

m/z (LCMS ES+, 70V) 472 (MNa<sup>+</sup>).

$\delta_H$  (d6-DMSO, 380K) 3.41 (4H, t, J7.2Hz), 3.31 (4H, s), 2.46 (4H, t, J7.2Hz) 1.44 (18H, s).

**Intermediate 5****2,3-Bis-*tert*-butoxycarbonylamino-succinic acid bis-succinimidyl ester**

To Intermediate 2 (1.500g, 4.31mmol) in dichloromethane (20ml) were added N-hydroxy succinimide (1.240g, 10.78mmol) and EDC (2.066g, 10.78mmol). After 5 overnight reaction the solution was diluted to 50ml with dichloromethane, washed with water (3x30ml), dried over magnesium sulphate and the solvent removed to give a white solid residue. The residue was purified by silica column chromatography eluting with 50-65% ethyl acetate in hexane to yield the desired di-NHS ester, 277mg, 12%, as a white solid.

10 m/z (LCMS ES+, 70V) 565 (MNa<sup>+</sup>).  
δ<sub>H</sub> (CDCl<sub>3</sub>-rotamers) 6.47, 6.17, 6.10 (2H, 3xbr), 5.35, 5.25, 5.10 (2H, 3xbr), 2.82 (8H, s), 1.44, 1.42 (18H, 2xs).

**Intermediate 6****{*tert*-Butoxycarbonyl-[2-(*tert*-butoxycarbonyl-carboxymethyl-amino)-ethyl]-amino}-acetic acid bis-succinimidyl ester**

To a suspension of Intermediate 3 (200mg, 0.53mmol) in dichloromethane (4ml) was added triethylamine (269mg, 2.66mmol) and once a clear solution was obtained, N-hydroxy succinimide (153mg, 1.33mmol) followed by EDC (255mg, 1.33mmol) were 20 added. LCMS of the reaction at 45min and 5h showed it to have stopped before completion. An extra 1.5 equivalents of both N-hydroxy succinimide and EDC were added in DCM (3ml) and the reaction left overnight during which time a poorly soluble white solid formed. The reaction mix was diluted to 40ml with dichloromethane, the solution/suspension washed with 0.1M HCl (6x50ml), dried over magnesium sulphate 25 and the solvent removed. The resulting white solid residue was purified by silica column chromatography eluting with 70% ethyl acetate in hexane to yield the desired di-NHS ester, 38mg, 13%, as a poorly soluble white solid.

m/z (LCMS ES+, 70V) 593 (MNa<sup>+</sup>).

δ<sub>H</sub> (d<sub>6</sub>-DMSO, 380K) 4.36 (4H, s), 3.46 (4H, s), 2.84 (8H, s), 1.45 (18H, s).

**Intermediate 7****3-(*tert*-Butoxycarbonyl-{2-[*tert*-butoxycarbonyl-(2-carboxy-ethyl)-amino]-ethyl}-amino)-propionic acid bis succinimidyl ester**

5 To Intermediate 4 (1.00g, 2.48mmol) in dichloromethane (40ml) were added triethylamine (751mg, 7.43mmol), N-hydroxy succinimide (712mg, 6.19mmol) and EDC (1.186g, 6.19mmol). After 2h20min a further 474mg of EDC was added and the reaction left overnight at ambient temperature. The solvent was removed and the residue purified by silica column chromatography, eluting 70% ethyl acetate in hexane to yield the  
10 product, 773mg, 52%, as a white solid.  
m/z (LCMS ES+, 70V) 621 (MNa<sup>+</sup>).  
 $\delta_H$  (d6-DMSO, 380K) 3.54 (4H, t, J7.0Hz), 3.37 (4H, s), 2.91 (4H, t, J7.0Hz), 2.83 (8H, s), 1.46 (18H, s).

15 **Intermediate 8**

**2,3-Bis-*tert*-butoxycarbonylamino-N,N'-bis-(4-maleimidyl-butyl)-succinamide**

To Intermediate 1 (300mg, 1.120mmol) was added 1:1 trifluoroacetic acid: dichloromethane (8ml). After 30 minutes the solvent was removed, the residue dissolved in dichloromethane and Intermediate 5 (276mg, 0.509mmol) added, followed  
20 immediately by triethylamine (258mg, 2.546mmol). After 30 minutes PS-TsCl scavenger resin (0.5g, 1.44mmol/g) was added, stirred for 1 hour and filtered off. The solvent was removed and the residue purified by silica column chromatography eluting with 75-100% ethyl acetate in hexane to give the desired material, 115mg, 35%, as a white solid.  
m/z (LCMS ES+, 70V) 649 (MH<sup>+</sup>).  
25  $\delta_H$  (CDCl<sub>3</sub>) 6.64 (2H, br), 6.62 (4H, s), 6.28 (2H, br), 4.30 (2H, br), 3.46 (4H, t, J6.9Hz), 3.17 (4H, m), 1.57-1.40 (8H, m), 1.39 (18H, s).

**Intermediate 9****(2-{*tert*-Butoxycarbonyl-[(2-(methoxy-polyethoxy)-ethylcarbamoyl)-methyl]-amino}-ethyl)-[(2-(methoxy-polyethoxy)-ethylcarbamoyl)-methyl]-carbamic acid *tert*-butyl ester**

To a solution of Intermediate 6 (5.7mg, 0.010mmol) in dichloromethane (5ml) were added 20K methoxy-PEG-amine (500mg, 0.025mmol) (purchased from Rapp

Polymere) and triethylamine (5.1mg, 0.050mmol). The reaction was left overnight, diluted with dichloromethane to 25ml and stirred for 3 days with MP-Tosic acid scavenger resin (3.0g, 1.43mmol/g). The resin was filtered off, the solution diluted to 50ml with dichloromethane and washed with 0.1M HCl (4x30ml). The solution was dried over magnesium sulphate and the solvent removed to give the 40K product, 370mg, 92%, as a waxy white solid.

5  $\delta_H$  (CDCl<sub>3</sub>) 4.1-3.3 (~3600H, brm), 3.30 (6H, s), 1.42 (18H, br).

### Intermediate 10

10 **(2-{*tert*-Butoxycarbonyl-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-ethyl}-amino)-ethyl-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-ethyl]-carbamic acid *tert*-butyl ester**

To a solution of Intermediate 7 (11.1mg, 0.019mmol) in dichloromethane (10ml) were added 20K methoxy-PEG-amine (1.00g, 0.05mmol) (purchased from Rapp Polymere) and triethylamine (9.4mg, 0.093mmol). The reaction was left overnight, diluted with dichloromethane to 50ml and stirred for 3 days with MP-Tosic acid scavenger resin (6.0g, 1.43mmol/g). The resin was filtered off, the solution washed with 0.1M HCl (4x50ml), dried over magnesium sulphate and the solvent removed to quantitatively yield the 40K product as a waxy white solid.

15  $\delta_H$  (CDCl<sub>3</sub>) 4.5-3.0 (~3600H, brm), 3.30 (6H, s), 2.4 (4H, br), 1.37 (18H, brs).

### Intermediate 11

#### **2,3-Diamino-N,N'-bis-[4-maleimidyl-butyl]-succinamide bis TFA salt**

20 Intermediate 8 (4.0mg, 0.0062mmol) was dissolved in dichloromethane (1ml) and trifluoroacetic acid (1ml) added. After half an hour the solvent was removed and the residue used crude in the synthesis of Example 1.

m/z (LCMS ES+, 70V) 449 (MH<sup>+</sup>).

25  $\delta_H$  (d6-DMSO) 6.93 (4H, s), 4.14 (2H, s), 3.32 (4H, t, J6.8Hz), 3.06, 2.96 (4H, 2xm), 1.42 (4H, m), 1.29 (4H, m).

**Intermediate 12****N-(2-(Methoxy-polyethoxy)-ethyl)-2-(2-[(2-(methoxy-polyethoxy)-ethylcarbamoyl)-methyl]-amino)-ethylamino)-acetamide di-hydrochloride**

Intermediate 9 (approx 400mg) was dissolved in dichloromethane (4ml), and 5 trifluoroacetic acid (4ml) added. After half an hour the solvent was removed, the residue dissolved in dichloromethane (100ml), washed with 0.1M HCl (3x100ml), dried over magnesium sulphate and the solvent removed to yield the salt (386mg) as an off-white solid.

$\delta_H$  (CDCl<sub>3</sub>) 4.2-3.0 (~3600H, brm), 3.31 (6H, s).

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**Intermediate 13****N-(2-(Methoxy-polyethoxy)-ethyl)-3-[2-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-ethylamino]-ethylamino)-propionamide di hydrochloride salt**

Intermediate 10 (460mg) was dissolved in dichloromethane (5ml), and 15 trifluoroacetic acid (5ml) added. After half an hour the solvent was removed, the residue dissolved in dichloromethane (60ml), washed with 0.1M HCl (4x60ml), dried over magnesium sulphate and the solvent removed to yield the salt (460mg) as a white solid.

$\delta_H$  (CDCl<sub>3</sub>) 7.20 (2H, br), 4.0-3.0 (~3600H, brm), 3.31 (6H, s), (remaining signals obscured by broad H<sub>2</sub>O signal).

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**Example 1****N,N'-Bis-[4-maleimidylbutyl]-2,3-bis-(3-(methoxy-polyethoxy)-propionylamino)-succinamide**

To Intermediate 11 (4.0mg, 6.2micromol) in dichloromethane (8ml) were added 25 M-PEG-SPA from Nektar (formerly Shearwater) (393mg, MW22K) followed by triethylamine (13mg, 123 $\mu$ mol). The reaction was stirred at ambient temperature for 3 days followed by 2 days at gentle reflux. To the reaction was added MP-TsCl resin (0.25g, 1.44mmol/g), the reaction gently stirred for 2 hours and the resin filtered off. The reaction was diluted to 50ml with dichloromethane, washed with 0.1M HCl (3x30ml), 30 dried over magnesium sulphate and the solvent removed. <sup>1</sup>H NMR showed the presence of unreacted M-PEG-SPA so the residue was dissolved in distilled water (10ml) and left overnight to hydrolyse the ester. The aqueous solution was washed with diethyl ether (2x50ml), extracted into dichloromethane (5x50ml), dried over magnesium sulphate and

the solvent removed to yield a white solid containing a mixture of mPEG-propionic acid, the mono-PEGylated species and the desired di-PEGylated species.

$\delta_H$  (CDCl<sub>3</sub>) 7.95 (2H, br), 7.55 (2H, br), 6.63 (4H, s), 4.89 (2H, br), 4.1-3.0 (~4000H, brm), 2.52 (4H, t), 1.58 (4H, m), 1.38 (4H, m).

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### Example 2

#### 3-Maleimidyl-N-(2-{|[3-maleimidyl-propionyl]-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-methyl]-amino}-ethyl)-N-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-methyl]-propionamide

10 Intermediate 12 (386mg, 0.0097mmol) was dissolved in dichloromethane (7ml) and to it added maleimido propionic acid N-hydroxysuccinimide ester (6.4mg, 0.024 mmol) followed by triethylamine (39mg, 0.386mmol). After overnight reaction the solvent was removed, the residue dissolved in dichloromethane (6ml) and half dropped into rapidly stirring diethyl ether (80ml) to give a white precipitate. This was filtered off  
15 under an atmosphere of nitrogen, dissolved in dichloromethane and the solvent removed to give a material with only partial maleimide addition. An 80mg batch of this material was retreated with 10 equivalents of maleimido propionic acid N-hydroxysuccinimide ester and 40 equivalents of triethylamine in dichloromethane (2ml) for 3 days, giving, after precipitation from diethyl ether, 80mg of a substantially unchanged product. This  
20 material was then treated with 10 equivalents of maleimido propionyl chloride (from maleimido propionic acid treated with 1:1 oxalyl chloride:dichloromethane (4ml) for 3 hours at ambient temperature) and 40 equivalents of triethylamine, giving, after precipitation from diethyl ether, material determined by <sup>1</sup>H NMR to consist of roughly a 1:1 mixture of the desired di-maleimide and the mono-maleimide species.  
25  $\delta_H$  (CDCl<sub>3</sub>) 6.65 (4H, m), 4.2-3.2 (~3600H, brm), 3.31 (6H, s), 2.57 (4H, m).

### Example 3

#### 3-Maleimidyl-N-(2-{|[3-(maleimidyl)-propionyl]-[2-(methoxy-polyethoxy)-ethylcarbamoyl]-ethyl]-amino}-ethyl)-N-[2-(2-(methoxy-polyethoxy)-ethylcarbamoyl)-ethyl]-propionamide

30 To maleimido propionic acid in dichloromethane (2ml) was added oxalyl chloride (2ml) and after 4.5 hours the solvent thoroughly removed under reduced pressure. To this crude acid chloride was added Intermediate 13 (116mg, 2.9 $\mu$ mol) in dichloromethane

(2ml) followed by triethylamine (12mg, 116 $\mu$ mol). After three days the solvent was removed, the residue redissolved in dichloromethane (3ml) and added slowly to rapidly stirred diethyl ether (100ml). The resulting white precipitate was filtered off, washed with diethyl ether, redissolved in dichloromethane and filtered. The solvent was then removed to yield the product in quantitative yield as a white, waxy solid.

5  $\delta_H$  (CDCl<sub>3</sub>) 6.81 (1H, t), 6.78 (1H, t), 6.69, 6.68 (4H, 2xs), 4.0-3.2 (~3600H, brm), 3.35 (6H, s), 2.72 (4H, m), (remaining signals obscured by broad H<sub>2</sub>O signal).

#### Example 4

10 **DiFab' Conjugate of Example 1 Product**

5ml of an engineered Fab' containing a single hinge thiol (see for example US 5,677,425; WO 98/25971), at 20mg/ml in 0.1M phosphate, 2mM EDTA, pH6, was reduced with a 20-fold molar excess of 2-mercaptoethylamine for 1 hour at ambient temperature. The reductant was then removed by gel filtration on five PD10 columns in 15 parallel equilibrated in 0.1M phosphate, 2mM EDTA, pH6. The concentration of the pooled reduced Fab' was determined by A280 (11.8mg/ml) and then 40mg of reduced Fab' was conjugated to 20mg of Example 1 (stock solution at 50mg/ml in 0.1M phosphate, 2mM EDTA, pH6) for 16 hours at ambient temperature. The extent of conjugation was assessed by SDS-PAGE and size-exclusion HPLC. To 3.33ml of the 20 Fab':PEG conjugation reaction was added 6.67ml of distilled water and 100 $\mu$ l of 1M acetic acid, and 10ml of this was loaded onto a 4ml SP-Sepharose HP C10 column (GE Healthcare) equilibrated in 50mM acetate, pH4.5, at 2ml/min. The column was eluted with a 16ml linear gradient of 0-250mM NaCl in 50mM acetate, pH4.5. 2ml fractions were collected and those containing PEG-DiFab' as assessed by SDS-PAGE were pooled. 25 The pooled fractions were concentrated with 10000 MWCO spin cartridges to 310 $\mu$ l. 300 $\mu$ l of this was loaded onto a Superose 6 HR10/30 gel filtration column (GE Healthcare) equilibrated in 50mM acetate, 125mM NaCl, pH5.5, at 0.5ml/min. The Superose 6 column was eluted with an isocratic gradient of 50mM acetate, 125mM NaCl, pH5.5, at 0.5ml/min. 0.5ml fractions were collected and those containing only 30 PEG-DiFab' (#B7-B5) as assessed by SDS-PAGE were pooled. The pooled fractions were concentrated with 10000 MWCO spin cartridges to 100 $\mu$ l. The concentration of the PEG-DiFab' was determined by A280 and the purity was assessed by reducing and non-reducing SDS-PAGE.

**Example 5****DiFab' Conjugate of Example 2 Product**

4ml of an engineered Fab' containing a single hinge thiol (see for example US 5,677,425; WO 98/25971) at 20mg/ml in 0.1M phosphate, 2mM EDTA, pH6, was reduced with a 20-fold molar excess of 2-mercaptoethylamine for 1 hour at ambient temperature. The reductant was then removed by gel filtration on four PD10 columns in parallel equilibrated in 0.1M phosphate, 2mM EDTA, pH6. The concentration of the pooled reduced Fab' was determined by A280 (12.0mg/ml) and then 22.7mg of reduced Fab' was conjugated to 4.8mg of Example 2 for 16 hours at ambient temperature. The extent of conjugation was assessed by reducing and non-reducing SDS-PAGE and size-exclusion HPLC. Conjugate purification and analysis as for Example 4.

**Example 6****DiFab' Conjugate of Example 3 Product**

4 ml of an engineered Fab' containing a single hinge thiol (see for example US 5,677,425; WO 98/25971) at 20mg/ml in 0.1M phosphate, 2mM EDTA, pH6, was reduced with a 20-fold molar excess of 2-mercaptoethylamine for 1 hour at ambient temperature. The reductant was then removed by gel filtration on four PD10 columns in parallel equilibrated in 0.1M phosphate, 2mM EDTA, pH6. The concentration of the pooled reduced Fab' was determined by A280 (14.6mg/ml) and then in duplicate 14.2mg of reduced Fab' was conjugated to 4mg of Example 3 (stock solution at 20mg/ml in distilled water) for 16 hours at ambient temperature. The extent of conjugation was assessed by SDS-PAGE and size-exclusion HPLC. The duplicate Fab':PEG conjugation reactions were pooled and to them was added 7.56ml of distilled water and 100µl of 1M acetic acid. 9.7ml of this was loaded onto a 4ml SP-Sepharose HP C10 column (GE Healthcare) equilibrated in 50mM acetate, pH4.5, at 2ml/min. The column was eluted with a 16ml linear gradient of 0-250mM NaCl in 50mM acetate, pH4.5. 2ml fractions were collected and those containing PEG-DiFab' as assessed by SDS-PAGE were pooled.

20 The pooled fractions were concentrated with 10000 MWCO spin cartridges to 260µl. 200µl of this was loaded onto a Superose 6 HR10/30 gel filtration column (GE Healthcare) equilibrated in 50mM acetate, 125mM NaCl, pH5.5, at 0.5ml/min. The Superose 6 column was eluted with an isocratic gradient of 50mM acetate, 125mM NaCl,

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pH5.5 at 0.5ml/min. 0.5ml fractions were collected and those containing only PEG-DiFab' as assessed by SDS-PAGE were pooled. The pooled fractions were concentrated with 10000 MWCO spin cartridges to 570µl. The concentration of the PEG-DiFab' was determined by A280 (3.5mg/ml) and the purity was assessed by 5 reducing and non-reducing SDS-PAGE, endotoxin assay and size-exclusion HPLC.